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INCORPORATION OF SHIKIMATE AND OTHER PRECURSORS INTO AROMATIC AMINO ACIDS AND PRENYLQUINONES OF ISOLATED SPINACH CHLOROPLASTS

HORST BICKEL*, LOTTE PALME* and GERNOT SCHULTZ†

*Institut für Botanik, and †Institut für Tierernährung, Arbeitsgruppe Phytochemie und Futtermittelkunde, Tierärztliche Hochschule, D 3000 Hannover, GFR

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Key Word Index—*Spinacea oleracea*; Chenopodiaceae; isolated intact chloroplasts; shikimate pathway; aromatic acids; plastoquinone; α-tocopherol.

Abstract—Under conditions of photosynthesis, shikimate-[1,6-^{14}C] and D,L-tyrosine-[β-^{14}C] were incorporated into the aromatic amino acids Phe, Tyr and Trp, and the prenylquinone and α-tocopherol by intact spinach chloroplasts. This might indicate the presence of enzymes of shikimate pathway in chloroplasts.

INTRODUCTION

Shikimate [1], tyrosine [2,3] and its metabolites [4,5] were incorporated into prenylquinones like PQ, in chloroplasts [6,7]. Further the transfer of Phe across the chloroplast membrane (envelope) occurred no faster in comparison to other amino acids [8]. However, chloroplasts were not necessarily automonous with respect to the synthesis of aromatic amino acids. Therefore, the present study was undertaken to clarify the role of chloroplasts in the synthesis of these amino acids via the shikimate pathway and the subsequent reactions in the formation of PQ and αT.

Comparing the method for isolation of intact chloroplasts, the different incorporation patterns of peak I and peak III chloroplasts isolated by Larsson *et al.* [9, 10] were taken into consideration. Fractionating a suspension of intact chloroplasts isolated according to Jensen and Bassham [11] with a two-phase-system, a large portion of peak-I chloroplasts (=intact chloroplasts without cytoplasm), a smaller portion of peak-III chloroplasts (=intact chloroplasts surrounded by a cytoplasm portion with mitochondria, ER, peroxisomes and cytoplasmic membrane, 'multiorganelle complexes' [12]) and broken chloroplasts were obtained. As compared to peak-I chloroplasts, peak-III chloroplasts incorporated a relatively high amount of photosynthetically fixed carbon into amino acids [9,10]. To exclude the influence of cytoplasm, intact chloroplasts isolated according to Jensen and Bassham [11] (=intact chloroplasts A) and those isolated according to Larsson *et al.* [9,12] (=intact chloroplasts B) were used.

RESULTS

The incorporation into amino acids, PQ and αT was studied by adding $^{14}CO_2$, shikimate-[1,6-^{14}C], D,L-tyrosine-[β-^{14}C], and homogentisate-[-^{14}C], respectively, to spinach leaves and intact chloroplasts isolated according to methods mentioned above (intact chloroplasts A and B) and conditions of photosynthesis. The dansyl-method [13] was used to separate amino acids from high amounts of other primary products of photosynthesis. By this the exact separation of aromatic amino acids (Fig. 2) from the more concentrated amino acids Ala, Ser, Glu, Gly etc. (Fig. 1) was achieved. The following snags were eliminated before shikimate and other precursors were applied for incorporation in chloroplast suspensions: (i) nitrogen depletion decreasing the synthesis of amino acids; (ii) influence of

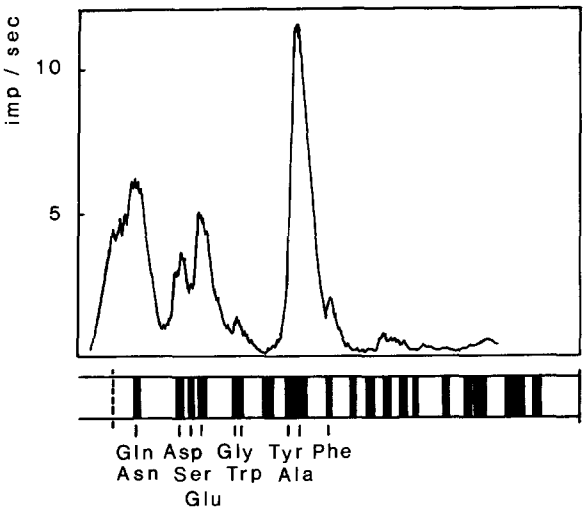

Fig. 1. Radioscan of the thin layer chromatogram of the dansylated amino acids obtained from a suspension of intact chloroplasts A exposed to $^{14}CO_2$. Experimental conditions: vol = 10 ml; 6 mg chlorophyll; 83 μmol $NaH^{14}CO_3$ [=200 μCi]. TLC on Si gel with C_6H_6–Py–HOAc (40:10:1) for incubation procedure and preparation of dansylated amino acids see Experimental.

Parts of the results were reported at the Int. Congr. Biochem., Hamburg 1976, and the Botaniker Tagung, Zürich 1976.

Abbreviations: PQ, plastoquinone; αT, βT, γT, δT, α-, β-, γ- and δ-tocopherol, respectively.

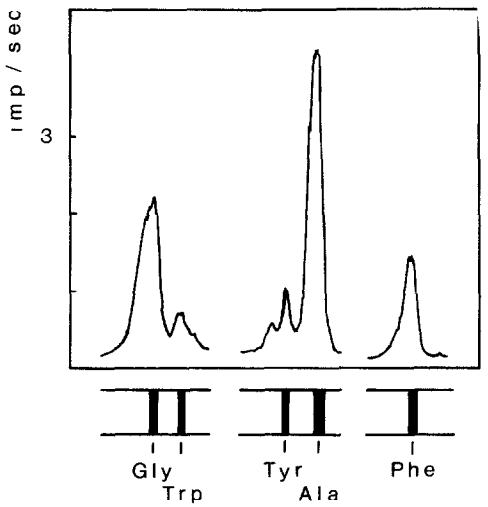

Fig. 2 Radioscan of dansylated amino acids in Fig. 1, rechromatographed repeatedly on layers of micropolyamide ('repeated chromatography') with H_2O–HCO_2H (100:1,5) and C_6H_6–HOAc (9:1) respectively. Each peak in Fig. 1 was prepared on a separate plate. From the Ala zone, a portion was cut away

microorganisms, contaminating the chloroplast suspension, on synthesis of amino acids.

Nitrogen supply for the synthesis of amino acids

To prove, if nitrogen was available in sufficient concentrations in the chloroplast suspension for synthesizing amino acids, KNO_2 used in optimal concentrations [R. Pflüger, personal communication] for nitrite reduction in chloroplasts [14–16], Glu and $GluNH_2$, respectively, were administered to the medium, described in Experimental. $GluNH_2$ and Glu were administered, although details concerning the primary acceptor for reduced nitrogen, substrates for transamination and other exchange reactions (e.g. reaction of chorismate with $GluNH_2$ to anthranilate; and reactions of indolglycerophosphate with Ser to Trp) have been known in microorganisms [17] but not in higher plants [18]. As can be seen in Table 1, the rate of photosynthetic fixation of carbon into amino acids is not increased by the supply of the above nitrogen compounds.

Extent of synthesis of amino acids by microorganisms contaminating the chloroplast suspension

To estimate the microbial synthesis of amino acids, possibly occurring in non-sterile chloroplast suspensions, the following experiments were carried out. In the first, the net synthesis of amino acids in the darkness from primary products of photosynthesis was investigated. Preilluminating a chloroplast suspension for 20 min, the amount of free and peptide bound aromatic amino acids did not increase in 20 min without light (Table 2). That was an indication for a synthesis of aromatic amino acids from primary products of photosynthesis neither by chloroplasts nor by microorganisms in the darkness.

A second study was carried out to incorporate shikimate-[1,6-^{14}C] into aromatic amino acids in the darkness. Table 5 shows that the incorporation in the darkness into Phe was less than one twentieth, that into Tyr *ca* one fifth, and that into Trp *ca* one fourth of those of the corresponding experiment in light. Disregarding the low rate of synthesis of aromatic amino acids, probably by chloroplasts in the darkness (compare the increase of petrol-phase in the darkness (Table 2)), the rate of microbial synthesis might be negligible. In addition only very small numbers of microorganisms could be detected with the electron microscope.

Table 1. Incorporation of $^{14}CO_2$ into the amino acids Ala, Phe, Tyr, and Trp by intact chloroplasts A after administration of KNO_2, Glu, and $GluNH_2$, respectively

	Without addition	2×10^{-4}M KNO_2	2×10^{-4}M Glu	2×10^{-4}M $GluNH_2$
	(dpm/mg chlorophyll) [in brackets: % photosynthetic fixed $^{14}CO_2$/mg chlorophyll × 30 min]			
Ala	68000 [0.14]	35000 [0.073]	55000 [0.12]	68000 [0.14]
Phe	7160 [0.015]	2490 [0.0052]	2930 [0.0061]	1025 [0.0021]
Tyr	1205 [0.0025]	780 [0.0016]	640 [0.0013]	770 [0.0016]
Trp	10000 [0.021]	20000 [0.042]	10000 [0.021]	20000 [0.042]

Each experiment: vol. = 2.35 ml; 1.3 mg chlorophyll; 13.3 μmol $NaH^{14}CO_3$ [= 200 μCi], photosynthetic $^{14}CO_2$-fixation 47.7×10^6 dpm/mg chlorophyll × 30 min; for incubation procedure see Experimental

Table 2. Incorporation of $^{14}CO_2$ into the amino acids and into PQ and αT by intact chloroplasts A after a period of 20 min light and 20 min light + 20 min darkness, respectively

	Intact chloroplasts A 20 min light	Intact chloroplasts A 20 min light + 20 min darkness (dpm/mg chlorophyll) [in brackets: % photosynthetic fixed $^{14}CO_2$/mg chlorophyll × 30 min]
Compounds in petrol (40–60°) phase	800000 [1.15]	1700000 [2.6]
Compounds in water phase	68900000 [98.9]	63900000 [97.4]
Sum	69700000 [100]	65600000 [100]
PQ	140 [0.0002]	1200 [0.0018]
αT	495 [0.0007]	780 [0.0012]
Ala	60000 [0.086]	220000 [0.335]
Phe	1935 [0.0028]	1790 [0.0027]
Tyr	2435 [0.003]	1405 [0.002]
Trp	465 [0.0007]	880 [0.0013]

Each experiment vol. = 1.3 ml, 1.1 mg chlorophyll, 11.7 μmol $NaH^{14}CO_3$ [= 100 μCi]; for incubation procedure see Experimental. Testing the hydrolysate (12 hr, 6M HCl, 100°), no radioactivity was found in peptides and proteins of chloroplasts, illuminated for 30 min.

Table 3. Distribution of Phe, Tyr, and Trp between chloroplasts and medium, investigated in a control experiment to Table 5 (2. Column: intact chloroplasts A; light)

	% in Supernatant	% in Pellet
Phe	92.0	8.0
Tyr	77.2	22.8
Trp	89.9	10.1

After illuminating for 30 min with 10 μCi shikimate-[1,6-^{14}C], the suspension was centrifuged (2 min at 2000 *g*) for fractionating into pellet (=chloroplasts and the surrounding portion of medium) and supernatant (=medium).

Distribution of aromatic amino acids between chloroplasts and medium

On average 80% of the radioactivity in the aromatic amino acids was found in supernatant (=medium) and 20% of that in sediment (= chloroplasts and a surrounding portion of medium) after rapid centrifuging (30 sec, 2°) a suspension of chloroplasts A, incubated for 30 min with shikimate-[1,6-^{14}C] in the light (Table 3). As mentioned earlier, synthesis by microorganisms appeared to be impossible and hence the distribution between chloroplasts and medium could be caused only by a transfer of amino acids, synthesized in the chloroplasts, across the envelope into the medium.

This agreed with results of [8] demonstrating the absorption especially of Phe by spinach chloroplasts, unlike that of Ala, Ser, Gly.

Table 4. Incorporation of $^{14}CO_2$ into PQ, αT, and the amino acids Ala, Phe, Tyr, and Trp by intact chloroplasts

	Intact chloroplasts A Light
	(dpm/mg chlorophyll) [in brackets: % photosynthetic fixed $^{14}CO_2$/mg chlorophyll × 30 min]
Compounds in petrol (40–60°) phase	5900000 [4.0]
Compounds in water phase	141000000 [96.0]
Sum	147300000 [100]
PQ	<
αT	<
Ala	90400 [0.031]
Phe	10000 [0.0034]
Tyr	1440 [0.0005]
Trp	20800 [0.0071]

Vol. = 1.3 ml; 0.5 mg chlorophyll; 11.7 μmol $NaH^{14}CO_3$ [=100 μCi); for incubation procedure see Experimental.

Incorporation of $^{14}CO_2$

The incorporation of $^{14}CO_2$ into the amino acids can be inferred from Figs 1 and 2. Throughout the experiments the ratio of incorporation into Ala : Phe : Tyr remained fairly constant, the values being for intact

Table 5. Incorporation of shikimate-[1,6-^{14}C] into PQ, αT, and the amino acids Ala, Phe, Tyr, and Trp by spinach leaves and intact chloroplasts

	Leaves Light	Intact chloroplasts A Light	Intact chloroplasts A Dark	Intact chloroplasts B Light
	(dpm/mg chlorophyll) [in brackets: % of added shikimate-[1,6-^{14}C] in dpm/mg chlorophyll]			
Compounds in petrol (40–60°)	14300000 [63.8]	2330000 [70.2]	1960000 [53.0]	3440000 [16.9]
Compounds in water phase	8090000 [36.2]	990000 [29.8]	1740000 [47.0]	16900000 [83.1]
Sum	22400000 [100]	3320000 [100]	3700000 [100]	20300000 [100]
PQ	19900 [0.09]	2570 [0.08]	654 [0.02]	247 [0.001]
αT	11300 [0.05]	8215 [0.25]	752 [0.02]	1400 [0.007]
Ala	3320 [0.015]	n.d.	n.d.	1470 [0.007]
Phe	99000 [0.44]	13540 [0.40]	1125 [0.03]	50635 [0.25]
Tyr	32270 [0.14]	3115 [0.09]	1265 [0.03]	17310 [0.09]
Trp	4430 [0.02]	10185 [0.31]	5290 [0.14]	26660 [0.13]

Shikimate-[1,6-^{14}C] [sp. act. 12.5 μCi/μmol] 10 μCi/expt. Spinach leaves: Excised leaves (1 mg chlorophyll) were placed in a cuvette (10 × 10 cm) with 1 ml vessels containing the labelled shikimate in 0.5 ml H_2O. The soln was vacuum infiltrated into the vascular system just before illuminating the cuvette for 120 min. CO_2 was supplied by a membrane pump. Intact chloroplasts A, light experiment: vol. = 10 ml; 6.7 mg chlorophyll; 80 μmol $NaHCO_3$. Dark experiment (6.0 mg chlorophyll) was prepared in the same manner, but the cuvette was completely darkened by enveloping with an alumina foil. Intact chloroplasts B. vol. = 1.3 ml; 1.1 mg chlorophyll; 10 μmol $NaHCO_3$; for incubation procedure see Experimental. n.d. = not determined.

chloroplasts A on the average 16.6:1:0.19 (compare Tables 1 and 4) and for intact chloroplasts B 19.9:1:0.12. Assuming a proportional incorporation of $^{14}CO_2$ into amino acids, the molar ratio of the labelled Ala:Phe:Tyr was 50:1:0.2. Hence in intact chloroplasts a synthesis of amino acids were evident (Ala *ca* 0.03%; Phe *ca* 0.003%; Tyr *ca* 0.0005% of the incorporated ^{14}C)—even after separation of multiorganelle complexes (peak-III chloroplasts [9]). The incorporation of $^{14}CO_2$ into PQ and αT using isolated chloroplasts A and B, respectively, was negligible (Table 4). These results agreed with those of earlier work [19] in which this was successful only after adding phenolic compounds.

Incorporation of shikimate-[1, 6-^{14}C]

Strikingly, the amounts of incorporation of shikimate-[1,6-^{14}C] and of D,L-tyrosine-[β-^{14}C] into PQ and αT were different, depending upon the method used for isolation of chloroplasts. In intact chloroplasts A, shikimate-[1,6-^{14}C] was incorporated into aromatic amino acids, PQ and αT whereas in intact chloroplasts B, it was incorporated only in amino acids. The leaves behaved as intact chloroplasts A (Table 5).

The negligible incorporation of the labelled shikimate into Ala indicated a small decomposition of shikimate. A microbial synthesis of aromatic amino acids tested in darkness could be ruled out (see above).

Incorporation of D,L-tyrosine-[β-^{14}C]

D,L-Tyrosine-[β-^{14}C] (Table 6) as a more direct precursor to PQ and αT could be incorporated in larger amounts than shikimate. The labelled tyrosine just as the labelled shikimate (Table 5) was incorporated into PQ and αT by intact chloroplasts A but not by chloroplasts B, though the photosynthetic fixation of carbon took place at normal rates in an experiment with $^{14}CO_2$ carried out in parallel.

Table 6. Incorporation of D,L-tyrosine-[β-^{14}C] into PQ and αT by intact chloroplasts

	Intact chloroplasts A Light	Intact chloroplasts A Light + Phe	Intact chloroplasts B Light
	(dpm/mg chlorophyll) [in brackets: % of added D,L-tyrosine-[β-^{14}C] in dpm/mg chlorophyll]		
Compounds in petrol (40–60°) phase	3750000 [41.5]	3810000 [42.1]	9730000 [77.3]
Compounds in water phase	5280000 [58.5]	5250000 [57.9]	3540000 [26.7]
Sum	9030000 [100]	10700000 [100]	13300000 [100]
PQ	166700 [1.85]	170000 [1.87]	630 [0.005]
αT	133000 [1.48]	81300 [0.90]	1050 [0.008]

D,L-Tyrosine-[β-^{14}C] [sp. act. 14.75 μCi/μmol] (10 μCi per experiment). Intact chloroplasts A, with and without Phe (1 μmol), respectively: vol. = 10 ml; 2.5 mg chlorophyll; 80 μmol $NaHCO_3$. Intact chloroplasts B. vol = 1.3 ml; 1.8 mg chlorophyll; 10 μmol $NaHCO_3$; for incubation procedure see Experimental.

Table 7. Incorporation of homogentisate-[α-^{14}C] into PQ and αT by spinach leaves, and intact chloroplasts

	Leaves Light	Intact chloroplasts A Light	Intact chloroplasts B Light
	(dpm/mg chlorophyll) [in brackets: % of added homogentisate-[α-^{14}C] in dpm/mg chlorophyll]		
Compounds in petrol (40–60°) phase	190000 [4.7]	770000 [8.7]	230000 [4.3]
Compounds in water phase	3860000 [95.3]	8120000 [91.3]	5050000 [95.7]
Sum	4050000 [100]	8900000 [100]	5290000 [100]
PQ	13900 [0.33]	238 [0.003]	190 [0.004]
αT	5800 [0.14]	121 [0.001]	51 [0.001]

Homogentisate-[α-^{14}C] [sp.act. 14.8 μCi/μmol]. Spinach leaves: 2.2 μCi; intact chloroplasts A: 4.4 μCi; Intact chloroplasts B 2.6 μCi. Spinach leaves: 1.2 mg chlorophyll; for further experimental details see Table 5. Intact chloroplasts A and B, respectively; vol. = 1.3 ml; 1.2 mg chlorophyll; 10 μmol $NaHCO_3$; for incubation procedure see Experimental.

Incorporation of homogentisate-[α-^{14}C]

Homogentisate-[α-^{14}C], being a precursor according to ref. [4], was incorporated into PQ and αT only by leaves, not, however, by intact chloroplasts A and B (Table 7), though homogentisate was kept in the reduced state by addition of ascorbic acid (proved by TLC of the supernatant of chloroplasts suspension).

DISCUSSION

The results of ^{14}C-incorporation experiments are summarized in Table 8. As shown in Table 5, the rates of incorporation from shikimate-[1,6-^{14}C] into the aromatic amino acids and PQ and αT were comparable in both cases, using isolated chloroplasts A and whole leaves. As seen in Table 6, tyrosine-[β-^{14}C] as a more direct precursor was incorporated into PQ and αT to a higher degree than shikimate-[1,6-^{14}C]. On the basis of these results, it could be concluded that preparation of intact chloroplasts were capable of synthesizing aromatic amino acids and prenylquinones starting from shikimate.

On the other hand, on using $^{14}CO_2$ under photosynthetic conditions (Tables 1 and 4), the incorporation rate into the above mentioned compounds in the chloroplasts was low in comparison to that of whole leaves. This was found to be true even for Ala (though it is a non-aromatic amino acid), throughout all chloroplast preparations, isolated by different methods.

To explain the discrepancies between the above results, two alternative hypotheses are proposed. (1) The synthesis of amino acids, especially the stages between the incorporation of CO_2 and the formation of shikimate, are dependent on a co-operation of chloroplasts with other compartments of the cell. (2) The low activity of chloroplasts is caused artificially by diluting out the substrates into the medium during photosynthesis. An alternative explanation for lowering

the activity is the loss of cofactors, both ions and coenzymes, or enzymes during the preparation procedure.

Let us examine the arguments for both the hypotheses. There is some evidence for the co-operation of chloroplasts with other organelles, hypothesis (1), in forming the intermediate products of shikimate pathway (compare [16, 18]). Nevertheless, the low over-all activity of amino acid synthesis could be explained, hypothesis (2), by the dilution of the substrates into the medium. Dihydroxyacetone phosphate, formed in large amounts as one of the primary products of photosynthesis, is transferred by the phosphate translocator [21, 22] at high rates [23] across the envelope. As the ratio of volumes of chloroplasts to medium was in the range of 1:100, large amounts of primary products of photosynthesis were transferred into the medium (according to ref. [10]: 98% of dihydroxyacetone phosphate, 80% of 3-phosphoglycerate, 70% of ribose-5-phosphate + ribulose-5-phosphate). Therefore it must be assumed that the concentration of the above substrates decreased by dilution to an extent far below the K_m-values of the enzymes for the subsequent reactions (e.g. for the formation of amino acids). Moreover, on using intact chloroplasts B, the inability to incorporate ^{14}C into PQ and αT might be attributed to a loss of substrates, cofactors and/or enzymes, perhaps from the area of the envelope, whereas intact chloroplasts A isolated in a short time were intact in respect to these reactions.

Table 8. Incorporation of ^{14}C from $^{14}CO_2$, shikimate-[1,6-^{14}C], D,L-tyrosine-[β-^{14}C], and homogentisate-[α-^{14}C] into PQ, αT, and amino acids (see also [19])

^{14}C Labelled precursor		^{14}C-Incorporation	
$^{14}CO_2$	leaf	non-aromatic amino acids and aromatic amino acids	PQ, αT
	chloroplasts A	non aromatic amino acids and aromatic amino acids	min amounts PQ αT
	chloroplasts B	non aromatic amino acids and aromatic amino acids	min amounts †
Shikimate [1,6-^{14}C]	leaf	aromatic amino acids	PQ, αT
	chloroplasts A	aromatic amino acids	PQ, αT
	chloroplasts B	aromatic amino acids	†
D,L-tyrosine [β-^{14}C]	leaf		PQ, αT
	chloroplasts A		PQ, αT
	chloroplasts B		†
Homogentisate [α-^{14}C]	leaf		PQ, αT
	chloroplasts A		†
	chloroplasts B		†

*After additional administration of tyrosine, *p*-hydroxyphenylpyruvate, and homogentisate, respectively.
†Incorporation rate was one hundredth up to one thousandth of that into leaves.

As shown in ref. [4], homogentisate was incorporated in large amounts into PQ, γT and αT by higher plants and algae. Although the results in the present work using leaves agreed with those of ref. [4], the experiments failed in using both intact chloroplasts, A and B. To clarify this discrepancy, further investigations should be done.

Strikingly, the incorporation into both PQ and αT of spinach chloroplasts, was in the same range. This was not in agreement with our former expts [24] on barley in which only a labelling of PQ and not of αT was found under photosynthetic conditions with $^{14}CO_2$. In recent investigations on lettuce (E. Keppler and G. Schultz, unpublished), a considerable incorporation from $^{14}CO_2$ into γT was however observed; this was further transformed partially into αT after an interval of 6 hr without $^{14}CO_2$. So the results obtained in barley may be interpreted as an indication for a regulation in the presence of $^{14}CO_2$ rather than an extraplastidic synthesis. Considering the partial extraplastidic distribution of γT after fractionating tissues of fruits of *Lycopersicum esculentum* [25], however, it might be inferred that a transfer of these tocopherols occurred across the envelope into the cytosol, and/or a synthesis took place in the area of the envelope.

EXPERIMENTAL

Radioisotopes. Homogentisate was prepared according to [4] from D,L-tyrosine-[β-^{14}C], catalyzed by an enzyme preparation of lyophilized rat liver [26, 27]. Shikimate-[1, 6-^{14}C] was obtained from CIS, Gif-sur-Yvette, France; $NaH^{14}CO_3$ and D,L-tyrosine [β-^{14}C] were purchased from Amersham–Buchler.

Isolation of intact chloroplasts. Leaves of local spinach were used for the isolation of chloroplasts. The preparation of intact chloroplasts A and intact chloroplasts B was performed as described in [11] and [9, 12], respectively.

Incubation procedure for intact chloroplasts. The intact chloroplasts were centrifuged and the pellet was resuspended in medium C pH 7.6 [11, see also 19]; for vol. of medium, addition of $NaHCO_3$, $NaH^{14}CO_3$, shikimate-[1,6-^{14}C], D,L-tyrosine-[β-^{14}C] and homogentisate-[α^{14}C], respectively, see legends in Figs and Tables. The suspensions (T = 20 ± 2°) were illuminated for 30 min with white light (1×10^6 erg min^{-2} sec^{-1}) in a polyacryl cuvette (d = 0.5 and 0.1 cm, respectively).

Isolation and determination of amino acids. 25 ml Me_2CO and 25 ml petrol (40–60°) were added to the chloroplast suspension. This soln was washed with 100 ml H_2O in a separating funnel. The H_2O–Me_2CO phase was acidified with M HCl to pH 2, and applied to a cation exchange column (Dowex W 50 × 8 in H^+-form). After washing with 50 ml H_2O the amino acids were eluted with 10 ml of 10% NH_3. The eluate was evapd under red. pres. The residue was dansylated according to [13] in 1 ml 0.1 M $NaHCO_3$ and 1 ml dansyl-Cl [1 mg/ml Me_2CO]. TLC of dansyl-amino acids [13] was carried out on Si gel with C_6H_6–Py–HOAc (40:10:1) the rechromatography was performed on layers of micropolyamide with H_2O–HCO_2H (100:1.5).

Isolation and determination of prenylquinones and β-carotene see refs [19, 24].

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